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# Clinical and Genetic Characterization of Patients with Arrhythmogenic Right Ventricular Dysplasia/Cardiomyopathy Caused by a Plakophilin-2 Splice Mutation

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## Key Words

Arrhythmia · Cardiomyopathy · Genetics · Desmosome · Arrhythmogenic right ventricular dysplasia/cardiomyopathy

## Abstract

**Objectives:** Arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) is characterized by fibrofatty replacement of cardiomyocytes. In around 50% of index patients, a genetic predisposition is demonstrated. The purpose of this study was to examine a plakophilin-2 (*PKP2*) splice site mutation, c.2489+4A>C, identified in 4 separately ascertained Dutch ARVD/C families. **Methods:** Genealogical studies and comprehensive screening of 5 desmosomal genes were undertaken. Reverse transcriptase PCR (RT-PCR) and subsequent sequencing was performed. **Results:** An A-to-C change (c.2489+4A>C) near the splice donor site of intervening sequence 12 of *PKP2* was found in all 4 families.

Based on pedigree data and haplotype sharing, a common ancestor should be situated more than 7 generations ago. RT-PCR demonstrated the presence of aberrant messenger RNA. Clinical manifestations ranged from severe disease to nonpenetrance in elderly mutation carriers. **Conclusions:** This founder mutation in *PKP2* is predicted to lead to the presence of a dysfunctional *PKP2* protein, whereas most truncating mutations are expected to lead to loss of protein. Mutation carriers displayed a wide range of disease severity, suggesting that *PKP2* mutations alone are not sufficient to cause disease, which results in the variable expression and incomplete penetrance characteristic of ARVD/C mutations.

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J.J.v.d.S. and P.A.v.d.Z. contributed equally to this article.  
F.G. passed away on May 15, 2008.

## Introduction

Arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) is a disorder characterized by progressive replacement of cardiomyocytes by fibrofatty tissue, primarily in the right ventricle. Life-threatening ventricular arrhythmias originating from the right ventricle can be an early manifestation of the disease. Clinical diagnosis depends on the fulfillment of Task Force Criteria (TFC) [1], for which modifications have recently been proposed [2].

An important genetic contribution is evident from the fact that in 30–50% of cases one or more first-degree relatives also display signs of the disease [3, 4]. A large majority of mutations in ARVD/C patients has been found in genes encoding different components of the cardiac desmosome, i.e. plakophilin-2 (*PKP2*), desmocollin-2 (*DSC2*), desmoglein-2 (*DSG2*), desmoplakin (*DSP*), and plakoglobin (*JUP*) [5–12], suggesting that ARVD/C is primarily a disease of disturbed desmosomal function [13, 14]. Mutations in other genes have also been reported in ARVD/C, i.e. among others, transmembrane protein 43 (*TMEM43*), desmin (*DES*), and titin (*TTN*) [15–18], indicating genetic heterogeneity. In addition, several ARVD/C cases were found to be caused by multiple mutations in the same gene (compound heterozygosity) or mutations in different genes (digenic inheritance), which could result in an earlier onset and increased disease severity [19–21]. The precise mechanisms by which mutations in these genes lead to the distinct ARVD/C phenotype remain, to a large extent, to be elucidated. Decreased desmosome numbers, structurally abnormal desmosomes, secondary effects on other intercalated disk structures, or impaired Wnt/beta-catenin signaling, resulting in increased expression of adipogenic and fibrogenic genes and fat accumulation, may all contribute to the ARVD/C pathophysiology [22–24]. However, regardless of the gene that is mutated, translocation of plakoglobin from the desmosome to the cytosol may be an important common step in disease causation [22, 24, 25].

It has been shown that mutations in *PKP2* are frequent in ARVD/C patients in the northwestern part of Europe and the USA [5, 7, 26, 27]. In a cohort of Dutch ARVD/C patients up to 90% of *PKP2* mutations were found in cases of proven familial disease [21]. Eight different recurrent *PKP2* mutations have been found in the Dutch population, accounting for 84% (41/49) of separately ascertained *PKP2*-positive ARVD/C families. For some of them a shared *PKP2* haplotype was demonstrated, while other families could be traced back to a common ancestor

by genealogy, sometimes as far as 10 generations ago [7]. Therefore, it is likely that several different *PKP2* founder mutations segregate in The Netherlands, which may contribute to the high prevalence of *PKP2* mutations in Dutch ARVD/C patients.

Looking at the spectrum of different mutations reported in *PKP2*, it is evident that truncating mutations (nonsense and frameshift) are most frequent [28]. The classification of missense variants as pathogenic, in *PKP2* as well as in other genes, remains challenging without solid functional evidence [29]. Truncating mutations are predicted to lead to loss of *PKP2* protein by nonsense-mediated messenger RNA decay (NMD), although mRNA studies by Gerull et al. [5] showed that the identified splice mutations had detectable mRNA with frameshifts, resulting in premature termination codons, that may evade NMD. As a general rule, introduction of a stop codon more than 50–55 nucleotides upstream of the last exon-intron boundary of any gene will lead to the breakdown of messenger-RNA in vivo, resulting in only very little translation of the abnormal truncated protein [30]. This is an argument for haploinsufficiency (insufficient amounts of normal *PKP2*) as the predominant mechanism by which *PKP2* mutations predispose to disease, as opposed to a dominant negative effect due to the presence of abnormal *PKP2* protein. Indeed, a Western blot from a heart biopsy from a patient with *PKP2* mutation c.2076\_2077delAA (p.Ser693CysfsX49) did not show detectable protein [5].

This report discusses both the clinical characteristics of patients from 4 different families that were found to have an identical splice mutation (c.2489+4A>C) at the C-terminal end of the *PKP2* gene, and the possible mechanism by which it exerts its effect.

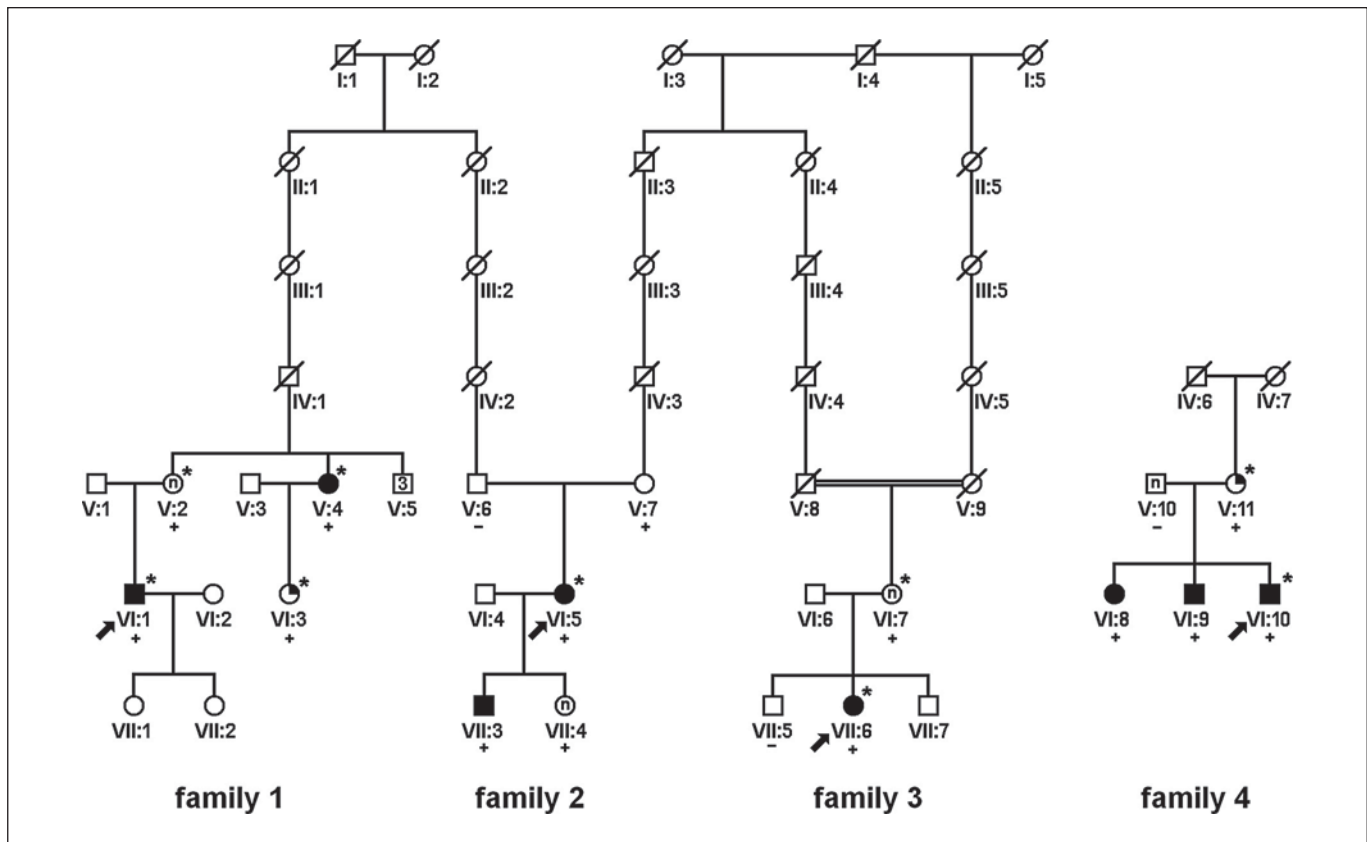
## Methods

### Clinical Evaluation

All index patients and their relatives were evaluated at the Cardiology Departments of the University Medical Centers in Utrecht and Amsterdam. Family members were evaluated following a genetic counseling procedure and after informed consent had been obtained. The evaluation consisted of at least a physical examination, 12-lead ECG, and echocardiography. Diagnosis was based on the recently modified ARVD/C TFC [2].

### Genealogy

Since 1997, genealogical studies have been performed in newly ascertained ARVD/C cases, in order to select patients that could contribute to the finding of new ARVD/C loci, using identity by descent strategies [31]. Investigations were carried out using community registries, starting from information on the grandparents as supplied by the index patients.



**Fig. 1.** Pedigrees of families 1–4. Squares denote males, circles females. Black squares/circles denote individuals that satisfy the 2010 TFC for a clinical diagnosis of ARVD/C ( $\geq 4$  points). Upper right quadrant-blackened squares/circles denote individuals that did not fulfill the TFC but did have abnormalities compatible with

ARVD/C at their most recent cardiac examination. +/– indicates the presence or absence of the *PKP2* c.2489+4A>C mutation. Individuals denoted with ‘n’ had a normal cardiac evaluation. Numbers in squares/circles denote the number of individuals. \* Genotype used for the haplotype sharing test, using SNP array data.

#### Molecular Genetic Analyses

DNA was isolated from peripheral lymphocytes according to standard protocols. In index patients the coding regions of the *PKP2*, *DSC2*, *DSG2*, *DSP*, *JUP*, and *TMEM43* genes were analyzed using denaturing high-performance liquid chromatography (*PKP2*, *DSC2*, and *DSG2*) as described previously [32], and/or direct sequencing (*DSP*, *JUP*, and *TMEM43*). Direct sequencing was performed with a BigDye Terminator DNA sequencing kit (version 2.0) on a 3730 automated sequencer (Applied Biosystems, Foster City, Calif., USA). All sequences were analyzed using SeqScape software (version 2.1.1, Applied Biosystems). In addition, multiplex ligation-dependent probe amplification analysis was performed to exclude large deletions in *PKP2* (SALSA multiplex ligation-dependent probe amplification kit P168 ARVC-*PKP2*; MRC Holland, Amsterdam, The Netherlands). Relatives of index patients were only analyzed for the presence of a previously identified mutation.

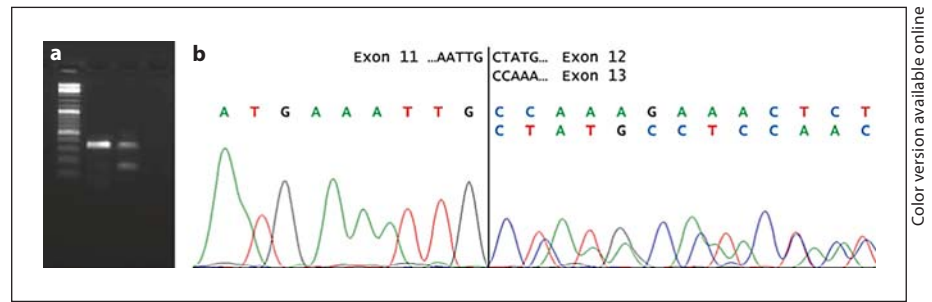
Total RNA was isolated from fresh blood samples from individual VII:6 and a control individual using a PAXgene kit (QIAGEN Benelux, Venlo, The Netherlands) and subjected to random hexamer primed reverse transcriptase PCR (RT-PCR).

The obtained cDNA products were amplified by PCR with primers specific for the *PKP2* coding sequence. *PKP2* cDNA fragments were separated according to size using 2% agarose gel electrophoresis along with a 100-bp DNA ladder (O’GeneRuler; Fermentas, Burlington, Ont., Canada). Both normal and aberrant fragments were gel purified and used for direct sequencing. Primers and PCR details are available upon request.

## Results

### Genealogy

In families 2 and 3 (fig. 1), common ancestors were found who were born around 1780 AD. Family 1 could be linked to family 2, but a link with the common ancestors in families 2 and 3 could not be established. Family 4 could not be traced back to the same ancestors, yet all ancestors lived in the same region.



**Fig. 2.** RT-PCR for the *PKP2* c.2489+4A>C mutation. **a** Two percent agarose gel electrophoresis showing cDNA products derived from mRNA of a control sample and index patient VII:6. The first lane shows a 100-bp ladder, followed by the control, the patient sample, and a blank with primers in exons 11 and 13. The expected normal cDNA product with primers in exons 11 and 13 is 335 bp in length. The shorter transcript in the patient sample (lane 3)

lacks exon 12 (190 bp) as shown in **b**. The bands in the patient sample show an ~1:1 ratio, indicating that preferred PCR of the shorter transcript, lacking exon 12, did not occur. This could indicate partial degradation of the mutated mRNA. **b** Sequencing of the cDNA derived from both the normally spliced transcript and the abnormally spliced transcript demonstrating the skipping of exon 12.

**Table 1.** Results of splice prediction programs

Splice prediction program	Wild type	c.2489+4A>C mutation	Notes
NetGene2	0.93	0.91	both ‘highly confident donor sites’ donor score cutoff: 0.40
NNSPLICE 0.9	0.98	0.57	
<i>Using Alamut mutation interpretation software</i>			
SpliceSiteFinder-Like	73.60	62.81	
MaxEntScan	9.73	6.22	
GeneSplicer	2.54	1.02	

Results of the different prediction programs for the normal exon 12 donor splice site are shown. Splice predictions are available from the following websites: NetGene2: [www.cbs.dtu.dk/services/NetGene2](http://www.cbs.dtu.dk/services/NetGene2), NNSPLICE 0.9: [www.fruitfly.org/seq\\_tools/splice.html](http://www.fruitfly.org/seq_tools/splice.html), MaxEntScan: [genes.mit.edu/burgelab/maxent/Xmaxentscan\\_scoreseq.html](http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), GeneSplicer: [www.cbc.umd.edu/software/GeneSplicer](http://www.cbc.umd.edu/software/GeneSplicer), and Alamut: [www.interactive-biosoftware.com](http://www.interactive-biosoftware.com).

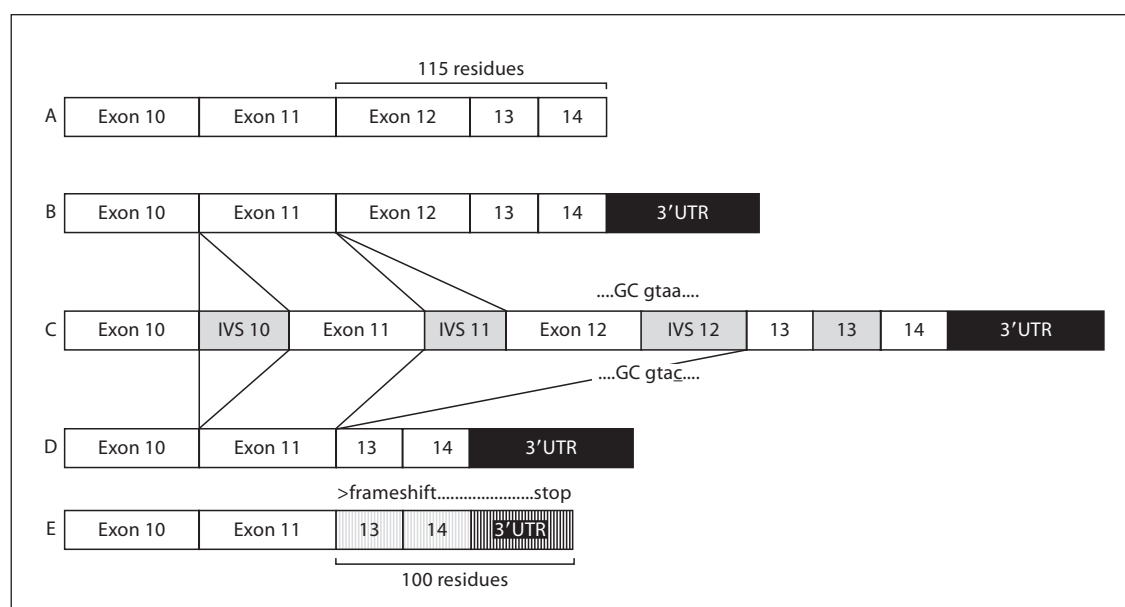
### Molecular Genetic Analyses

Analysis of the entire coding regions of *PKP2*, *DSC2*, *DSG2*, *DSP*, *JUP*, and *TMEM43* including exon-intron boundaries in the 4 index patients revealed a c.2489+4A>C mutation in the *PKP2* gene. Besides this intervening sequence mutation, no other mutations, including large deletions in *PKP2*, were found. The *PKP2* c.2489+4A>C mutation is within the splice donor consensus sequence. Splice prediction programs were inconclusive with respect to a predicted effect on splicing (table 1). The mutation was absent in over 150 ethnically matched controls (300 alleles) and not annotated in both dbSNP and the 1000 Genomes Project databases (ac-

cessed October 10, 2011). In addition to the 4 index patients, 10 relatives were also found to carry the *PKP2* c.2489+4A>C mutation.

RT-PCR demonstrated the presence of an abnormal *PKP2* messenger RNA (fig. 2a), indicating that the effect of the c.2489+4A>C mutation is indeed through aberrant splicing. The abnormal *PKP2* messenger RNA lacks the entire exon 12 (190 bp) (fig. 2b). In the predicted protein a frameshift occurs that leads to stop codon 99 residues downstream, well within the 3' untranslated region of the *PKP2* gene. Therefore, all residues normally encoded by exons 12, 13, and 14 are missing from the abnormal transcript, but the predicted abnormal protein is only





**Fig. 3.** Aberrant splicing as a result of the *PKP2* c.2489+4A>C mutation. A and B represent the C-terminus of the normal plakophilin-2 protein and the normally spliced mRNA, respectively. C depicts part of the genomic DNA of the *PKP2* gene [exons in white, introns (IVS) in grey, 3' untranslated region (UTR) in

black] with the position of the wild-type splice site above the bar and the mutated splice site below the bar. D and E show the aberrant splice product and the predicted abnormal protein, respectively. The abnormal protein is only 15 amino acid residues shorter than the wild-type protein.

15 residues shorter than the wild-type protein (fig. 3). Since the stop codon occurs in the 3' untranslated region, the abnormal protein cannot be subjected to NMD.

### Clinical Evaluation

All index patients with the *PKP2* c.2489+4A>C mutation fulfilled the modified TFC for ARVD/C [2]. Figure 1 shows the pedigree of all families, and clinical data on the *PKP2* c.2489+4A>C mutation carriers are summarized in table 2. Index patient 1 (VI:1) was admitted to the hospital at the age of 33 years with sustained monomorphic ventricular tachycardia (VT) (240/min) with left bundle branch block (LBBB) morphology during exercise. He experienced at least 4 other episodes of VT, but no syncope. Supraventricular tachycardias also occurred, caused by AV nodal re-entry and provoked by premature ventricular complexes. Radiofrequency (RF) ablation was performed for the ventricular arrhythmias from the right ventricular outflow tract and for the AV nodal re-entry tachycardia (AVNRT). He has been doing well on disopyramide medication for 12 years. Index patient 2 (VI:5) has been known to have frequent VT episodes with LBBB morphology since the age of 20, which were treated with RF ablation. At the age of 36 she had an out-of-hospital

cardiac arrest (OHCA) due to ventricular fibrillation (VF) while on sotalol. Subsequently an implantable cardioverter defibrillator (ICD) was implanted, which has delivered appropriate anti-tachycardia pacing and shocks. Index patient 3 (VII:6) experienced syncope at the age of 26 during exercise. She was admitted with a wide QRS complex tachycardia (250/min). Treatment with disopyramide was started. An AVNRT was diagnosed at electrophysiological study, which was treated with RF ablation, after a second arrhythmia episode. She has been free of symptoms for 14 years. Index patient 4 (VI:10) had an OHCA with VF at the age of 29. He received an ICD and was treated with metoprolol for VTs. At the age of 33 he experienced two episodes with electrical storms possibly triggered by a viral infection. Metoprolol was changed to sotalol and amiodarone was added. The patient has been doing well for 9 years.

In addition to the 4 index patients, 2 of the mutation-positive family members developed clinical complaints likely related to ARVD/C, resulting in 6 symptomatic mutation carriers. In family 4, a 34-year-old male (VI:9), who had had no complaints at the initial evaluation, received an ICD after he suffered a near syncope while already taking sotalol. Nonsustained polymorphic VTs of

**Table 2.** Clinical data on *PKP2* c.2489+4A>C mutation carriers

Individual (fig. 1)	Sex	Onset age years	Age at first evalua- tion years	Follow- up years	Events	Modified task force criteria for the diagnosis of ARVD/C								Treatment	Other
						func- tion	tissue <sup>a</sup>	repolar- ization	depolar- ization	arrhyth- mias	family history	task force criteria <sup>b</sup>	clinical status		
Family 1															
VI:1 <sup>c</sup>	M	33	33	12	VT	+				++	++	5	Aff	RF ablation, disopyramide	atypical AVNRT, no progression
V:2	F		61	0	none						++	2	Pos	none	no follow-up
V:4	F		69	7	none			++		++	++	6	Aff	anticoagulant therapy	paroxysmal AF, no progression
VI:3	F		35	7	none				+		++	3	Bor	none	no progression
Family 2															
VI:5	F	20	20	32	VT/VF/ OHCA	++		++	++	++	++	10	Aff	RF ablation, ICD, sotalol	appropriate ICD~, good LV function
VII:3	M		17	11	none	++			+		++	5	Aff	none	mild progression
VII:4	F		15	11	none						++	2	Pos	none	no progression
V:7	F														not evaluated
Family 3															
VII:6 <sup>d</sup>	F	26	26	14	syncope	+		++	+	++	++	8	Aff	RF ablation	concurrent AVNRT, no progression
VI:7	F		62	3	none						++	2	Pos	none	no progression
Family 4															
VI:10	M	29	29	9	VF/ OHCA	++		++		++	++	8	Aff	ICD, sotalol, amiodarone	appropriate ICD~, mild progression
V:11	F		59	9	none						++	2	Pos	none	mild progression
VI:8	F	40	33	9	near syncope	++					++	4	Aff	ICD	evident progression
VI:9	M	34	32	9	near syncope					++	++	4	Aff	ICD, sotalol	mild progression

AF = Atrial fibrillation; Aff = affected; Bor = borderline; F = female; ICD~ = ICD discharge; LV = left ventricle; M = male; NA = not available; nsVT = nonsustained ventricular tachycardia; Pos = possibly; RV = right ventricle; sVT = sustained ventricular tachycardia.

<sup>a</sup> Not available. <sup>b</sup> Minor criteria yield 1 point and major criteria 2 points; for a definite diagnosis of ARVD/C a minimum of 4 points is required, 3 points indicate a borderline diagnosis, and 2 points indicate a possible diagnosis. <sup>c</sup> For this patient a biopsy was available; it showed fibrosis but no fat and was considered inconclusive. <sup>d</sup> Syncope probably resulting from AVNRT; thus far no symptoms have recurred after RF ablation of AVNRT.

insufficient duration to warrant ICD therapy were subsequently recorded. His sister (VI:8) received an ICD shortly after she experienced a near syncope during a stressful event. Although without any signs of disease at initial evaluation at the age of 33, she developed aneurysmatic changes of the right ventricle in the course of 9 years.

Remarkably, 7 of the 13 mutation carriers (age range at first evaluation 15–69 years) who were evaluated were asymptomatic. In 4 of them only a major criterion for a positive family history was available, leading to a possible clinical status according to the modified TFC. In 3 of the asymptomatic carriers additional criteria were present, resulting in a borderline diagnosis in 1 and a definite diagnosis in 2 asymptomatic carriers [2].

Left ventricular (LV) involvement was not systematically assessed in these families. Cardiac MRI was performed in 5 mutation carriers; all of them showed normal LV volumes and LV ejection fraction. None of them

showed LV wall motion abnormalities or late gadolinium enhancement. Index patient VI:5 experienced VT with RBBB morphology, but she had a normal LV function. Finally, none of the mutation carriers had isolated inverted T-waves in the left precordial leads V4–V6.

## Discussion

*PKP2* mutations are a frequent cause of ARVD/C. The *PKP2* gene was first implicated in ARVD/C through a candidate gene approach [5]. The fact that the *PKP2* locus had not been previously found in linkage studies can be explained by low penetrance resulting in phenotype-negative family members who were classified as unaffected, although they were in fact mutation carriers. Previously, the haplotype sharing test identified a shared haplotype on chromosome 12p12.1–12q13.13, encompassing *PKP2*,

as the largest shared haplotype in these families [33]. The haplotype sharing test compares the genotypes from SNP arrays, using an affected-only strategy, to search for large shared haplotypes within pedigrees most likely to contain a causative mutation. In these families, the haplotype sharing test indicated a common founder, who must have lived well before the 19th century. Based on pedigree data, it was estimated that the most recent common ancestor should be situated over 7 generations ago. However, these are crude estimations since only 4 families were available. The actual age of the mutation could be considerably older. Little is known about the age of *PKP2* mutations. Our data show that mutations may have occurred many generations earlier, in the absence of a family history of cardiac disease. The fact that in some families we have been able to demonstrate common ancestors as far as 10 generations back by genealogy suggests that reproductive fitness in ARVD/C is not significantly reduced.

The *PKP2* c.2489+4A>C mutation seems to be relatively frequent in The Netherlands, having been detected in 4 independently ascertained ARVD/C families so far. Another 5 mutations (c.235C>T, c.397C>T, c.1211-1212dupT, c.2386T>C, and c.2489+1G>A) in *PKP2* occurred at least as frequently in the Dutch ARVD/C cohort. Haplotype analysis and genealogy suggest that there may also be common founders contributing to these mutations, explaining the high prevalence of *PKP2* mutations in Dutch ARVD/C patients [32, 34].

The frequent occurrence of nonsense and frameshift mutations in *PKP2* is indicative that the causative mechanism at the protein level is that of loss of normal *PKP2* function, as most of these mutations are predicted to lead to NMD [28]. Gerull et al. [5], while performing Western blot analysis on cardiac tissue in a patient with a *PKP2* c.2076\_2077delAA mutation, demonstrated reduced wild-type *PKP2* but were unable to demonstrate the predicted abnormal protein. This is in keeping with the concept of NMD in the heart. As the *PKP2* c.2489+4A>C mutation cannot be subjected to NMD, it is predicted to lead to a dysfunctional *PKP2* protein. However, we cannot exclude degradation of the abnormal protein through other mechanisms, which could still lead to haploinsufficiency. If a dysfunctional protein is present, the last two conserved armadillo repeat regions would be disrupted, completely altering the C-terminal tail. The exact role of the C-terminal tail is unknown, while the N-terminal part of the protein is most important for binding other desmosomal proteins and targeting *PKP2* to the plasma membrane [35]. The pathogenic effect of splice mutations

may be ameliorated when the induced aberrant splicing is not absolute, as has been shown for the only recessive *PKP2* mutation thus far [36]. In the absence of heterozygous polymorphisms in exons 12–14 of the *PKP2* gene, the presence of a normal transcript from the diseased allele could not be assessed.

In total 14 patients with the *PKP2* c.2489+4A>C mutation (4 males, 10 females), including index patients, were identified in the 4 families. Despite the overrepresentation of female mutation carriers in this study, 2 out of 4 index patients were males. Nine out of 10 family members with a mutation had a cardiac workup. Only 2 of 9 evaluated mutation-positive family members developed clinical complaints (table 2). Five family members fulfilled the modified TFC for ARVD/C. Incomplete penetrance and clinical variability are characteristic for ARVD/C [4] and were also seen in the families with the *PKP2* c.2489+4A>C mutation. Compound heterozygosity or digenic inheritance for mutations in the desmosomal genes or in *TMEM43* had been excluded as an explanation for this variability, but other genetic, epigenetic, or nongenetic factors, such as lifestyle, could have contributed to the clinical differences observed in these families.

Which preventive treatment modalities are justified in asymptomatic *PKP2* mutation carriers is currently undetermined. We suggest that they be followed-up regularly in order to assess disease progression with annual echocardiography, Holter monitoring, and exercise testing. Besides, additional cardiac MRI at intervals no longer than 5 years (or before implantation of an ICD) is deemed appropriate in The Netherlands.

Although we find molecular diagnosis in ARVD/C to be a useful tool in identifying persons at risk for developing ARVD/C, its value is still limited by the inability to accurately predict phenotypes from genotypes, especially when a missense variant is identified [29]. The fact that many identified *PKP2* mutation carriers will never experience life-threatening arrhythmias emphasizes the need for identifying the truly pathogenic mutations as well as other genetic or environmental factors that play a role in disease causation. We believe that the described *PKP2* c.2489+4A>C mutation is pathogenic, based on its cosegregation with the ARVD/C phenotype, the absence of the mutation in controls as well as in the dbSNP, and the 1000 Genomes Project databases, the fact that the mutation is located on the largest shared haplotype, indicating a founder effect [33], and the RT-PCR which demonstrated the presence of an abnormal *PKP2* messenger RNA (fig. 2a). As is the case for many other desmosomal muta-



tions implicated in ARVD/C, the c.2489+4A>C mutation is characterized by incomplete penetrance, indicating that not all mutation carriers will develop the disease. The mutation is predicted to lead to the presence of a dysfunctional PKP2 protein, whereas most truncating mutations are expected to lead to loss of protein. Mutation carriers displayed a wide range of disease severity, suggesting that *PKP2* mutations alone are not sufficient to cause disease.

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